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Upholding the T cell immune-regulatory function of CD31 inhibits the formation of T/B immunological synapses *in vitro* and attenuates the development of experimental autoimmune arthritis *in vivo*

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ABSTRACT

CD31, a trans-homophilic inhibitory receptor expressed on both T- and B-lymphocytes, drives the mutual detachment of interacting leukocytes. Intriguingly, T cell CD31 molecules relocate to the immunological synapse (IS), where the T and B cells establish a stable interaction.

Here, we show that intact CD31 molecules, which are able to drive an inhibitory signal, are concentrated at the periphery of the IS but are excluded from the center of the IS. At this site, were the cells establish the closest contact, the CD31 molecules are cleaved, and most of the extracellular portion of the protein, including the trans-homophilic binding sites, is shed from the cell surface.

T cells lacking CD31 trans-homophilic binding sites easily establish stable interactions with B cells; at the opposite, CD31 signaling agonists inhibit T/B IS formation as well as the ensuing helper T cell activation and function. Confocal microscopy and flow cytometry analysis of experimental T/B IS shows that the T cell inhibitory effects of CD31 agonists depend on SHP-2 signaling, which reduces the phosphorylation of ZAP70.

The analysis of synovial tissue biopsies from patients affected by rheumatoid arthritis showed that T cell CD31 molecules are excluded from the center of the T/B cell synapses *in vivo*. Interestingly, the administration of CD31 agonists *in vivo* significantly attenuated the development of the clinical signs of collagen-induced arthritis in DBA1/J mice.

Altogether, our data indicate that the T cell co-inhibitory receptor CD31 prevents the formation of functional T/B immunological synapses and that therapeutic strategies aimed at sustaining CD31 signaling will attenuate the development of autoimmune responses *in vivo*.

1. Introduction

A variety of receptors bearing the immunoreceptor tyrosine-based inhibitory motif (ITIM) are expressed by T cells, and each receptor plays a crucial and nonredundant role in immunoregulation

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[1]. CD31 is an ITIM receptor [2,3] constitutively expressed by T cells [4,5].

Although initially identified as adhesion molecules [6], CD31 receptors actually exhibit low avidity and transient "adhesive" properties, which correspond to the transhomophilic engagement that is necessary to trigger the outside-in signaling function of this receptor [7]. The trans-homophilic engagement of opposing CD31 molecules contributes to co-clustering of immunoreceptor tyrosine-based activation motif (ITAM) receptors through cis-homophilic interactions, provided that the latter are stimulated [7,8]. The proximity of CD31 molecules to ITAM receptors facilitates the phosphorylation of CD31 ITIMs, as CD31 does not exhibit intrinsic kinase activity [9].

The archetypal formation of the immunological synapse (IS) involves the co-clustering of ITAMs on T lymphocytes with other transmembrane receptors on the plasma membrane surface. The supramolecular activation cluster (SMAC) is in intimate contact with MHC molecules on the cognate antigen-presenting cell (APC), and T cell receptor (TCR) concentrations within this IS are indispensable for physiologically activating T cells. The SMAC comprises three regions: 1) the central SMAC (cSMAC), which contains TCR microclusters; 2) the peripheral SMAC (pSMAC), where adhesion molecules are concentrated, which favors synapse stability; and 3) the outmost part, called the distal SMAC (dSMAC), which contains large molecules, such as CD43 and CD45. Upon IS formation, the cytoskeleton of the cell is specifically organized, as reflected by the rapid and robust repositioning (docking) of the microtubule organizing center (MTOC) underneath the T cell plasma membrane and at the cSMAC. The docking of the MTOC allows the microtubule transport of vesicles containing effector molecules (e.g., cytokines or lytic molecules) to be directed toward the bound APC for subsequent polarized secretion. The effective activation of TCR signaling in the cSMAC requires the recruitment and phosphorylation of ZAP70 to the TCR ITAM, which is represented by the CD3ζsubunit. On the other hand, the presence of CD40L in the cSMAC drives CD40-dependent signaling in the APC [10]. In the case of T/B cell synapses, this interaction leads to B cell proliferation, class switch recombination, somatic hypermutation and the production of soluble IgM and IgG by B cells [11]. All of these activating signals at the IS must be tightly balanced by immunoregulatory signals to avoid inappropriate immune responses. Typically, T/B cell interactions are critically involved in rheumatoid arthritis (RA) [12]. Remarkably, CD31 knockout mice develop hyperactive T [13] and B [14] cell responses and are more sensitive to collagen-induced arthritis (CIA) [15,16].

The ITIM receptor CD31 is gaining much attention due to its immunoregulatory properties and for its peculiar trans-homophilic receptor engagement [17]. Interestingly, both T cells and professional APCs, including B cells, express CD31, which exerts important co-inhibitory functions upon trans-homophilic interactions [14,18]. Although it has been shown that CD31 molecules relocate to the interface between T and B cells upon stimulation with a superantigen [19], neither the spatial distribution of CD31 across the SMAC nor its potential regulatory role in the IS is known.

The fact that CD31 co-clustering with the TCR can inhibit $CD4^+$ T cell activation [18] suggests that this ITIM receptor exerts a regulatory role in IS formation. However, we recently showed that the cleavage and shedding of CD31 occurs rapidly upon T cell activation following antibody-mediated TCR crosslinking [20]. Furthermore, these processes abrogate potential CD31 inhibitory signaling and can explain why the regulatory role of CD31 in T cell activation is transitory [18].

Herein, we show that intact and functionally inhibitory CD31 molecules accumulate on T cells at the periphery of the IS but are excluded from the cSMAC. The trans-homophilic portion of CD31, which is located at the most membrane-distal Ig-like domains, is selectively missing in the cSMAC, where the CD31 molecules appear truncated upon effective T/B IS formation. The absence of intact CD31 molecules in the cSMAC favors the stable formation of cell–cell contacts between T/B cell conjugates, which are prevented by intact CD31 molecules [21] and concomitantly facilitate TCR-mediated T cell activation. The latter mechanism is based on the results obtained in the present study, in which the use of a synthetic peptide to sustain ITIM signaling downstream of CD31 [20] dampened the rate of IS formation, the degree of T cell activation and their helper function toward B cells. Moreover, we show that T cell CD31 molecules are excluded also from the center of the T/B cell synapses occurring in the inflamed joints of patients with rheumatoid arthritis and that the administration of CD31 agonists in experimental mice significantly reduces the development of collagen-induced arthritis.

2. Materials and methods

Detailed method protocols are provided in the Supporting Material

2.1. Cells, reagents and antibodies

Jurkat and Raji cells were cultivated in RPMI-1640 Glutamax™ medium supplemented with 10% fetal calf serum and 50 μM β-mercaptoethanol. The synthetic CD31 peptide was prepared and used as previously described [20]. Recombinant CD31 protein was from R&D Systems and sodium stibogluconate from Merck-Millipore. The following antibodies were used: CD31 (clone JC70A, epitope within domain 1, DAKO); CD31-PE (clone MCB78.2, epitope within domain 6, Life Technologies); CD31 (goat polyclonal IgG, R&D Systems), CD31 (clone SP38, Spring Bioscience); CD3-V450®, SHP2 pY542-PE, ZAP70 pY292-PE, ß-tubulin-Cy3™, ERK pT202/pY204-PE, CD69-PE-Cy™7, CD19-AlexaFluor 700®, CD154-PE (all from BD Biosciences); CD20 (clone L26, DAKO); CD3 (clone F7.2.38, DAKO); and CD4 (clone SP35, Spring Bioscience). Immunofluorescence staining of mouse ankle joints was performed using a rabbit polyclonal anti-CD3 antibody (DAKO) and two anti-mouse monoclonal antibodies directed against the extracellular (clone MEC 13.3, BD Biosciences) and intracellular (clone SP38 Spring Bioscience) portions of mouse CD31. Fluorescently labeled Fcγ fragment-specific AffiniPure® $F(ab')_2$ fragments from Jackson ImmunoResearch Laboratories were used to detect positive staining of unconjugated primary antibodies. Soluble IL-2 (Human IL-2 FlexSet) and CD31 (as previously described [20]) were measured with the CBA® technology (BD Biosciences).

2.2. Jurkat–Raji conjugates

Raji cells were pre-incubated with 100 ng/ml staphylococcal enterotoxin E (SEE, Toxin Technology) prior to mixing the cells with Jurkat cells on polylysine-coated coverslips. Intracellular staining was carried on fixed (PFA) and permeabilized (0.5% Triton X-100) cells. For flow cytometry the conjugates were formed in solution and cells were distinguished by pre-labeling with either CFSE or CellTrace™ Violet (Life Technologies). Primary human FACS-purified $CD19^+$ cells were pre-incubated with a cocktail of superantigens (SEE, SEA and TSSP-1, 100 ng/ml) prior to mixing with $CD4^+$ cells.

2.3. Synovial tissue

Synovial tissue biopsies from inflamed synovial sites were obtained through arthroscopy from six RA patients. The protocol (00/

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